

# Adult Stem Cells and Skeletal Muscle Regeneration

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**Abstract:** Satellite cells are unipotent stem cells involved in muscle regeneration. However, the skeletal muscle microenvironment exerts a dominant influence over stem cell function. The cell intrinsic complexity of the skeletal muscle niche located within the connective tissue between fibers includes motor neurons, tendons, blood vessels, immune response mediators and interstitial cells. All these cell types modulate the trafficking of stimuli responsible of muscle fiber regeneration. In addition, several stem cell types have been discovered in skeletal muscle tissue, mainly located in the interstitium. The majority of these stem cells appears to directly contribute to myogenic differentiation, although some of them are mainly implicated in paracrine effects. This review focuses on adult stem cells, which have been used for therapeutic purposes, mainly in animal models of chronic muscle degeneration. Emerging literature identifies other myogenic progenitors generated from pluripotent stem cells as potential candidates for the treatment of skeletal muscle degeneration. However, adult stem cells still represent the gold standard for future comparative studies.

**Keywords:** Adult stem cells, muscular dystrophy, skeletal muscle regeneration.

## MUSCLE MICROENVIRONMENT AND INFLAMMATION: AN INTRODUCTION

Skeletal muscle is a complex tissue formed by multinucleated syncytial myofibers surrounded by a vast heterogeneity of environment-dependent cell types. Motor neurons, blood vessels, tendons and bones co-exist/colocalise with the interstitial cells, located within the connective tissue among fibers, to modulate the trafficking of stimuli between fibers and connective tissue. Moreover, growth factors, as well as secreted molecules and immune response mediators control physiological skeletal muscle homeostasis. Changes in cellular composition of muscle microenvironment are crucial for metabolic modifications occurring in acute muscle damage, chronic degeneration and regeneration. These processes greatly rely on the activation of Satellite Cells (SCs) and their dynamic interaction with the surrounding environment. This interplay takes place over different time scales. During acute events, such as following muscle trauma, it contributes to regeneration after temporary atrophic conditions. During chronic events, such as long lasting inflammatory processes affecting muscle in genetic diseases (muscular dystrophies or skeletal muscle myopathies) and in cancer-induced muscle atrophy and sarcopenia, this interplay is still critical to recover muscle function. In acute

and chronic skeletal muscle injury, Chemokine Ligand 1 (CXCL1) or CXCL5, released by mechanically stressed muscles, contribute to activation of immune responses. The first wave of inflammatory cells arriving at the site of injury within two hours after damage is composed of neutrophils [1]. Neutrophils enter the site of injury where they release reactive oxygen species also cytokines which then cause secondary tissue damage and recruit monocytes from the blood respectively [2]. Interestingly, when integrin beta-2 (CD18) is not expressed, neutrophil-recruitment is impaired [2] whereas resident macrophages responsible for the innate immune response to injury are still present [3]. Circulating monocytes, originating from the spleen reach the site of injury in a second wave of inflammatory cells and consequently differentiate into macrophages [4].

In muscular dystrophies (MDs), a heterogeneous group of inherited diseases characterized by the primary wasting of skeletal muscle, the functions of proteins that form a link between the cytoskeleton and the basal lamina are affected by mutations. Duchenne muscular dystrophy (DMD) is the most severe MD due to mutations in the gene encoding dystrophin (DMD). Affected patients usually die prematurely due to respiratory or cardiac failures [5]. The mdx (muscular dystrophy x-linked) mouse, carrying a nonsense point mutation in dystrophin exon 23 is the most used animal model for DMD.

Together with the increased fibrosis, a common hallmark of dystrophic muscles, distinct populations of macrophages have also been described in the muscle of mdx and affected individuals. Macrophages are a heterogeneous population

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and they can be functionally polarized into M1 (proinflammatory macrophages, classically activated) and M2 (alternatively activated) [6]. Macrophages exposed to Interferon- $\gamma$  or Tumor Necrosis Factor (TNF)- $\alpha$  are termed M1 macrophages, whereas those exposed to Interleukin (IL)-4, IL-13 and IL-10 are classified as M2 [7]. While M1 macrophages decline in numbers between day 2 and day 4 after acute muscle damage, high levels of M2 macrophages characterize subsequent tissue repair [7]. During acute muscle injury, the presence of M2 macrophages has been reported [8] but a clear understanding of their specific function is still missing. M2 macrophages most likely represent two heterogeneous populations that differ in time and localization [9]. These cells, expressing inducible nitric oxide synthase (iNOS), produce TNF- $\alpha$  and IL-1 $\beta$  cytokines. When the inflammation process progresses to resolution, macrophages start to express anti-inflammatory markers such as IL-10 or TGF $\beta$ , supporting the myogenic and angiogenic programs and favoring deposition of Extra Cellular Matrix (ECM) [9]. The transition between these two types of macrophages is still under investigation. However, phagocytosis of necrotic muscle cells and releasing of high levels of Interferon- $\gamma$  (IFN- $\gamma$ ) and TNF- $\alpha$  are associated with the M1 phenotype [10].

T lymphocytes that can differentiate in Th1 and Th2 also have defined roles in muscle regeneration. While Th1 inflammatory stimuli are responsible for neutrophils and macrophages recruitment required to clear cell debris, the Th2 immune response sustains the polarization of M2 macrophage and promotes skeletal muscle healing [11]. Delay in muscle regeneration with increased infiltration of CD8<sup>+</sup> T cells is observed in mice deficient for Cbl-b ubiquitin ligase [12].

Mast cells can also actively modulate skeletal muscle repair, since they have been shown to accumulate in the *gastrocnemius* muscle 8 hours after injury. Interestingly, they are present in mdx muscle tissue close to capillaries and have been linked to fibrosis [13]. Since their early appearance at the site of inflammation, this cell type is known to produce pro-inflammatory molecules, i.e. TNF- $\alpha$ , IL-6 and IL-1. Despite this function, a possible role in macrophage polarization remains to be elucidated.

In four-week-old mdx mice, M2 macrophages contribute to decreasing M1 cells and play a pivotal role in SC activation and induction of skeletal muscle regeneration [14]. Thus, improved therapy that relies on the manipulation of specific inflammatory cells is needed [15]. Nevertheless, the complexity of the mechanisms regulating the different subpopulations of cells during inflammation and muscle repair, together with fibrosis still need further elucidation. In this review, we will describe different muscle associated cell types (Fig. 1 and Table 1) that, together with SCs, populate skeletal muscle and are involved in the process of muscle tissue repair. Finally, we offer an overview of the association of genetic and cellular interventions to treat muscle regeneration impairment during pathologic conditions.

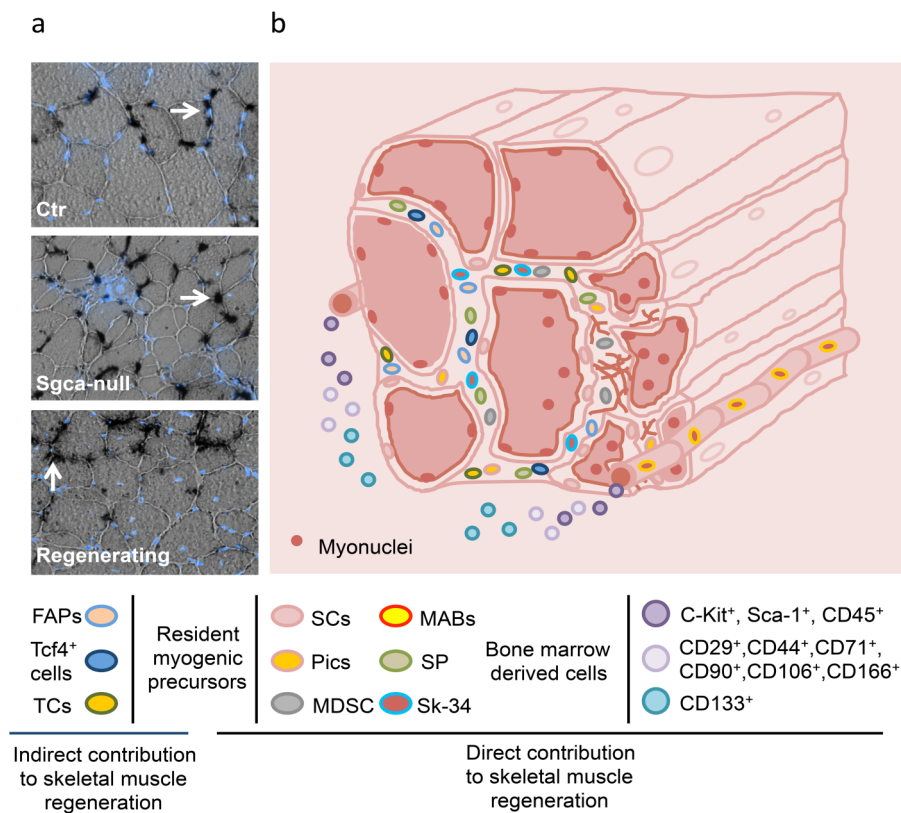
## SKELETAL MUSCLE MODULATING CELLS

### Satellite Cells (SCs)

In the adult, almost half of the body weight is represented by striated skeletal muscle tissue. This tissue is able to main-

tain shape and functionality thanks to the occasional fusion of muscle precursor cells (MPCs) with existing damaged fibers. It is worth noting that with exception for the head muscle, all trunk and limb muscle cells originate from an embryonic somite source [16]. Indeed, some years ago, Pax3 expressing precursors were reported for both skeletal muscle and smooth muscle cells in the embryonic dorsal aorta, suggesting a common origin before myogenic specification [17]. In contrast, cranial paraxial mesoderm is the original tissue for almost all of the head muscles, branchiomic and extraocular muscles [18].

SCs are located in the adult between the basal lamina and sarcolemma of muscle fibers [19] as quiescent unipotent stem cells. When activated by muscle damage, SCs enter the cell cycle as MPCs and contribute to the formation of new muscle fibers. SCs are distinguishable by their tightly wrapped shape and the disproportion between their cytosol with few organelles and a nucleus. The SC nucleus contains condensed chromatin, clearly smaller than the fiber myonuclei, due to the quiescence and to the transcriptionally poor activity of these cells [20]. SCs are highly abundant in oxidative slow muscles, where they are up to six times more numerous than in fast-glycolytic muscles. In slow muscle, they constitute between 2 and 7% of adult muscle nuclei and are usually located near blood capillaries [21] or in the proximity of the neuromuscular junction. The localization in specific anatomical structures and exposure to the signaling niche is directly implicated in SC self-renewal. Indeed, SCs can undergo both symmetrical and asymmetrical division, mainly depending on the location of their daughter cells with respect to the myofiber [22]. Enzymatic digestion and/or physical trituration allows SC release from the myofibers, and several protocols of expansion under specific proliferative conditions have been documented [23]. To note, the conditions used for the *in vitro* culture, i.e. specific proliferating medium containing growth factors and collagen-coated dishes, are responsible of the activation of SCs, as shown by the completely distinct gene expression profile of these cells in culture compared with the transcriptome of freshly isolated SCs [24]. A deeper understanding of the molecular features of these cells *in vivo* could be useful to better monitor and influence the quiescent or the activated and proliferating state of these cells in culture. Protocols for isolation by fluorescent-activated cell sorting (FACS) have been largely suggested, taking advantage of the extended panorama of molecular markers expressed by these cells. Among the expressed surface markers, cell adhesion protein M-cadherin [25], Desmin [26], tyrosine receptor kinase c-Met [27], chemokine receptor CXCR4 [28],  $\beta$ 1-integrin, syndecan-3 and syndecan-4 (two main transmembrane heparan sulfate proteoglycans) [29], caveolae-forming protein caveolin-1 [30] and calcitonin receptor [31] are now commonly accepted. CD34 [32] or the cell surface receptor  $\alpha$ 7-integrin [33], are not specifically expressed by SCs in skeletal muscles, and need to be taken in consideration always in combination with other specific markers. Among the nuclear markers, Pax7 is the canonical biomarker for quiescent and proliferating SCs [34], as well as envelope proteins lamin A/C and emerin, while the Notch ligand Jagged-1 could be used as a marker of activated SCs. The expression of Pax7, neuronal cell adhesion molecule (NCAM, also named CD56, [35])



**Fig. (1).** A. Alkaline phosphatase (AP) staining in murine *tibialis anterior* muscles from healthy control, *Sgca*-null (6 month old mice) and regenerating muscle (1 week after cardiotoxin injection). The vast of majority of interstitial cells are AP positive (black staining). Nuclei were stained in blue with Hoechst. B. scheme of degenerated skeletal muscle with the contribution of both resident myogenic precursors and bone marrow derived cells.

**Table 1.** List of adult stem cells contributing to skeletal muscle regeneration. \*Those cells (indirect contributors) contribute to muscle regeneration via paracrine effects.

Cell Type	Identification Markers		Reference
	Positive	Negative	
Satellite cells (SCs)	Pax7, M-cadherin, Desmin, cMet, CX-CR4, Syndecan3/4, CD34, Jagged-1	CD45, Sca-1, cKit	[26, 34, 28-30]
Mesoangioblasts (MABs)	AP, NG2, cKit, Flk-1, Sca-1, CD140, CD146	CD45, CD34, CD56, CD144, Pax7	[50, 52-54, 59, 119]
Interstitial cells (PICs)	PW-1, CD34, Sca-1	Pax7, Pax3	[62-64]
Muscle-Derived Stem Cell (MDSCs)	Flk-1, Sca-1, Desmin,	CD45, M-cadherin, CD34	[65, 66]
Side population (SPs)	Sca-1/ABCG2, Syndecan4, Pax7	CD45, CD43, cKit, CD11, Gr-1, B220, CD4, CD8	[68, 69]
Skeletal muscle-derived CD34+/45- (Sk-34)	CD34	Pax7, CD45, CD73	[75, 135]
Fibro-adipogenic progenitors (FAPs)	CD34, Sca-1, CD140	CD45, Lin, CD31, $\alpha$ -7 integrin	[78-80]
Telocytes (TCs)	ckit, CD34, PDGFRa	CD45, Pax7	[81]
Fibroblasts Tcf4+ (TCs)	Tcf/L2	CD45	[84]
Bone marrow cells	CD45, cKit, Sca-1, CD34	Lin	[137]
Mesenchymal stem cells	CD29, CD44, CD90, CD76, CD166, CD106, CD71, CD73, CD105	CD45, CD34, CD14, CD133	[46, 139]

and c-Met has been shown also in human SCs. Nevertheless, the heterogeneity of human SCs is high possibly due to the retainment of a more quiescent stage [32]. Accordingly, two SC populations have been identified by their mitotic rate measured by continuous BrdU labeling. One population is responsive and enters the cell cycle as the major population, while a reserve population, accounting for 20% of the entire population, maintains the quiescent state [36]. Other gene expression studies from SCs of different origins (limb and head muscles from both adult and aged mice) asserted that despite a different gene expression profile, SCs transplanted *in vivo* adopt a specific behavior according to microenvironment features [37]. The heterogeneous nature of the population is also supported by the asymmetric distribution of Numb in some but not all the SCs. Numb is a cell fate determinant and an inhibitor of the Notch signaling, the distribution of which contributes to the asymmetric division of SCs [38]. This is in agreement with the idea that the preferential and hierarchical retention of parental chromosomes from the most quiescent cells can avoid the accumulation of mutations in the DNA strands, probably acquired from the daughter cell during replication. A second hypothesis defends the nonrandom segregation of chromatids, with their respective epigenetic state, as the main reason for different gene expression pattern and cell fate. In this second view, one cell corresponds to the satellite stem cell state and the other to the satellite progenitor state [39]. Nevertheless many questions are still pending regarding the variety of markers used to isolate SCs and their function regarding the cell behavior. Given their strong myogenic commitment, SCs were thought as a major candidate for the treatment of muscular degeneration. However, pioneering experiments in mouse models showed poor survival and migration of transplanted SCs [40]. Although encouraging results have been obtained in Phase I clinical trials, SC treatments are hampered by the inability of those cells to cross the endothelial barriers, limiting their use to their direct intramuscular introduction.

### Bone Marrow Cells

Bone marrow mesenchymal stem cells (BM-MSCs) possess self-renewal ability and multilineage differentiation not only to mesoderm lineages, like chondrocytes, osteocytes and adipocytes, but also to ectodermic cells and endodermic cells [41, 42]. The peculiar characteristic and clinical relevance of MSCs is their ability to release trophic chemokines and growth factors that alter the local environment, facilitating replacement by local progenitors, promoting differentiation and angiogenesis, and exerting anti-apoptotic and immunomodulatory properties [43]. In the last 10 years, several studies have demonstrated how MSCs can promote myogenic regeneration. The first evidence was reported in 1998, showing that, the transplantation of genetically-modified bone marrow cells into immunodeficient mice, facilitated the migration of BM-MSCs into an area of injured muscle promoting regeneration and myogenic differentiation of the damaged fibers [44]. Gussoni *et al.* in 1999 demonstrated that wild-type (wt) total BM-MSCs or side population [BM-SP] cells achieved regenerating skeletal muscle fibers when transplanted into dystrophic mice [45]. In 2005, Dezawa *et al.* [46] showed that MSCs forced to express Notch and injected in mdx muscles resulted in a better recovery of dys-

trophin expression. Further studies will be necessary to elucidate the interactions between MSCs and the inflammatory milieu, the fate of the implanted cells and beneficial effects into damage tissue.

### Pericytes

Although discovered more than twenty years ago as cells surrounding capillaries and able to promote both angiogenesis and tissue repair, pericytes have recently received more attention. Indeed, pericytes were initially discovered for their ability to counteract and regulate blood flow [47]. In the last 15 years, these cells have been isolated from a large variety of tissues as cells expressing neural-glial-2 chondroitin sulphate proteoglycan (NG2; [48]) and Platelet Derived Growth Factor- $\beta$  (PDGF- $\beta$ ) receptor, critical for pericyte survival and development [49]. Regarding the expression of  $\alpha$ -Smooth Muscle Actin (SMA), there remain some concerns since pericytes in culture express SMA however, only a small percentage of pericytes *in vivo* are SMA positive [48]. In human, pericytes have been described as being positive for annexin V, alkaline phosphatase, desmin,  $\alpha$ -SMA, vimentin and to have high levels of PDGF receptor- $\beta$ , while, interestingly, they do not express other markers such as M-cadherin, N-CAM, cytokeratins and neurofilaments nor endothelial markers (such as CD31, CD34) or hematopoietic markers (CD45; [50]). Recently, a new marker profile - CD133<sup>+</sup>, CD34<sup>+</sup> and CD56<sup>+</sup> and CD45<sup>-</sup>, has been identified for the simultaneous isolation of pericytes, mesenchymal stem/stromal cells and blood derived stem cell subpopulations from human samples [51].

### Mesoangioblasts (MABs)

MABs can be found in the interstitial space between the muscle fibers in normal healthy tissue and their presence is altered in muscle pathologic conditions. Originally MABs have been isolated from dorsal aorta in the mouse embryo at day 9.5 of development, and more recently from adult muscle tissue of mice, dogs and humans [52-54]. Embryonic MABs express CD34/ c-Kit/ Flk-1 but are negative for NKX2.5/ Myf5/ Oct4 [55]. They differentiate to multiple mesodermal lineages *in vitro* and *in vivo* [56]. In the adult mouse and in dogs, MABs are usually extracted from skeletal muscle explants as cells positive for Alkaline Phosphatase (AP), Stem cell antigen-1 (Sca-1), NG2 proteoglycan, CD140a and CD140b [5, 50, 53, 54]. Adult MABs are multipotent and can differentiate toward myogenic, osteogenic, chondrogenic and adipogenic lineages [57]. Finally, human MABs also display CD146/ CD140b1/ NG2 but do not present any marker of hematopoietic or SCs (CD45<sup>-</sup>/ CD34<sup>-</sup>/ CD56<sup>-</sup>/ CD144<sup>-</sup>/ Pax7<sup>-</sup>; [50]). To note, a human correspondent to the murine Sca-1 marker is still missing although members of human Ly6 proteins have been proposed to have homologous functions [58]. Intriguingly, some markers expressed from human MABs are also characteristic of mesenchymal stem cells (CD10/ CD13/ CD44/ CD73/ CD90) and supporting the idea of a common origin for mesenchymal and pericyte stem cells [59]. Interestingly, a comparison among human MSCs, MABs and multipotent adult progenitor cells has confirmed a huge difference between the transcriptomes of these cell populations reflecting their specific functional properties and were just partially recovered by

culture conditions [60]. Several proinflammatory genes, cytokines and cytokine receptors are also expressed by MABs. Indeed, in a recent paper, the exposure to proinflammatory cytokines was shown to play an important role in MAB-induction of immunomodulatory cytokines able to inhibit T-cell proliferation *in vitro* [61]. This aspect is not negligible, opening the possibility for MABs to undergo new clinical applications to modulate immune responses.

### Pw1 Expressing Interstitial Cells (PICs)

PICs are also located in the interstitium among muscle fibers and they are characterized by the expression of PW1 transcription factor [62]. Analogously to previous cell types considered in this review, PICs are Pax7<sup>+</sup>/Sca-1<sup>+</sup> and CD34<sup>+</sup> and have been discovered embryonically as cells not related to SCs, since they do not have Pax3 myogenic progenitor cells, but are probably even able to originate them [63]. Transgenic mice carrying the N-terminal portion of PW1 under the control of a myogenin promoter regulatory sequence present normal muscle development in embryonic and fetal stages, but, postnatally, these mice show impairment in muscle development reflecting some features observed in Pax7<sup>-/-</sup> mice [64]. As multipotent myogenic progenitors, PICs are able to differentiate to skeletal and smooth muscle, but further studies are needed to better understand their origin and their similarity to MABs.

### Muscle Derived Stem Cell (MDSC)

MDSCs were isolated from the adult muscle tissue based on the expression of Flk1, Sca-1 and Desmin and on the absence of CD45, M-cadherin and CD34 [65]. These cells seem to have a different origin from the known SCs since they can be successfully isolated from Pax7<sup>-/-</sup> mice [66] and, as other cells populating the interstitial space among the myofibers, have been demonstrated to differentiate into myogenic, adipogenic, osteogenic, chondrogenic and even hematopoietic lineages [67]. Moreover a low expression of Major Histocompatibility Complex (MHC)-1 and consequently the inability to trigger infiltration of activated lymphocytes could be promising features for a more successful transplantation of these cells [65]. Once again it seems that those cells are strongly connected to MABs and consequently to PICs.

### Side Population (SP)

The term 'side population' (SP) is usually referred to a small subpopulation of stem cells present in adult muscle tissue and bone marrow, isolated for their ability to exclude DNA-binding dye (Hoechst 33342). SP cell limiting dilution experiments have demonstrated that SPs are able to reconstitute the entire bone marrow of mice lethally irradiated so that SPs are able to take different fates in permissive conditions [45]. Other groups have identified these cells as positive for surface markers like Sca-1/ABCG2 transporter, Syndecan-4 and Pax7 [68] but negative for hematopoietic stem cell markers, including CD43, CD45, c-kit, or immune cell markers, such as CD11, Gr-1, B220, CD4 and CD8 [69]. Although SPs have somite origins [70], they appear to be different from SCs, since still present in Pax7<sup>-/-</sup> mice [34]. In the recent literature, improvements of the expansion of these

cells have been generated and tested, letting free floating SP cells grow in suspension on a myoblast monolayer anchored on the surface of microcarriers [71]. Moreover, miR128a was found at high levels in SP cells and it was able to maintain the quiescent state in SP cells [72].

### SK-34 Cells

The interstitial space among the fibers has been reported as populated by two different cell fractions, such as CD34<sup>+</sup>/CD45<sup>-</sup> cells, also called Sk-34, and from CD34<sup>+</sup>/CD45<sup>-</sup> (Sk-DN; [73]). Sk-34 is a non-adherent myogenic cell type, not able to express Pax7, at least at the isolation, and is characterized by proliferation after muscle damage [74]. Moreover these cells, once injected beneath the renal capsule, present intrinsic plasticity for differentiation into muscle, pericytes, smooth muscle and peripheral nerve cells [75]. In addition, these cells are able to secrete nerve and vascular growth factors [76] making Sk-34 cells suitable as supportive paracrine mediators for nerve autografts. This interplay among the other interstitial stem cell types needs further investigation.

### Fibro/Adipocyte Progenitors (FAPs)

Fibroblasts are also present in the interstitial space among muscle fibers and they contribute to the extra cellular matrix deposition. Fibroblasts, can deposit collagen, laminin, fibronectin, tenascin, NCAM and Heparan Sulfate Proteoglycans (HSPGs). It has been reported that SCs can undergo differentiation to adipocytes or fibroblasts *in vitro* [77]. However, recent studies indicate that muscular adipocytes and fibrocytes present under pathological conditions may arise from FAPs at various stages of differentiation [78, 79]. FAPs were isolated from two independent groups as CD34<sup>+</sup>/Sca-1<sup>+</sup>/Lin<sup>-</sup>/CD31<sup>-</sup>/CD45<sup>-</sup>/α7integrin<sup>-</sup> [78] or as Sca-1<sup>+</sup>/CD140a<sup>+</sup>/α7integrin<sup>-</sup> [79] and they exert paracrine positive effects on muscle fibers. Interestingly, both groups demonstrated that these cells do not display myogenic capacity. Their positive action is stimulated when Histone Deacetylase (HDAC) inhibitors are used to promote follistatin secretion [80]. Certainly, it would be interesting to investigate the embryonic counterpart of these cells to better clarify their origin and their role in muscle embryonic development. Moreover, it would be of great importance to understand which chemokine/ growth factor/ endocrine compounds they produce in healthy conditions. Further studies should later be performed in order to also isolate the human counterpart for FAPs. Once these goals have been achieved, it may be attractive to use these cells for the treatment of pathologic conditions.

### Telocytes (TCs)

TCs, also referred to as interstitial Cajal-like cells (ICLCs), are a recently discovered cell type that populates the muscle interstitium. These cells are localized next to capillary and nerve endings, in close relationship with myofibers, SCs and other cell types. TCs express the proliferation marker c-kit and are able to transduce intercellular signaling via exosomes, ectosomes and multivesicular cargoes [81]. TCs are PDGFRβ<sup>+</sup>/CD34<sup>+</sup> cells and electron and light microscopy analysis identify their small body (9-15 μm) and a certain number of telopodes organized into networks with

other cells [82]. Their main function would be the maintenance of tissue homeostasis and through VEGF secretion they also stimulate vasculogenesis [83].

#### **Tcf4<sup>+</sup> Fibroblasts**

The transcription factor Tcf4 (transcription factor 7-like 2; Tcf7l2) is strongly expressed in fibroblast like cells localized in the connective tissue. They have been demonstrated using genetic manipulation of Tcf4 (GFPCre) mice to regulate muscle fiber type development and maturation [84]. In addition, low levels of Tcf4 in myogenic progenitor cells promote maturation of muscle fibers. Further characterization will be useful to understand the origin of these cells and their specific role in skeletal muscle regeneration.

### **MUSCLE DAMAGE AND REPAIR**

The modulation of muscle mass and multinucleated fiber size depends on protein and myonuclear turnover in case of muscle damage. However, the relative role of myogenic progenitors in muscle adaptation and hypertrophy is still questionable and, according to some authors, muscle hypertrophy does not require a *de-novo* fusion of cells [76]. Usually, minor lesions caused by spontaneous eccentric contractions, wear and tear or small injuries are repaired without the activation of inflammatory responses or cell implication. Nevertheless, the presence of Ca<sup>2+</sup> with intracellular vesicles and dysferlin-enriched patches are necessary, as demonstrated by the dystrophic-like phenotype in dysferlin-null mice [77]. Indeed, as evidenced by limb-girdle muscular dystrophy type 2B [78, 79] and type 1C [80] in patients harbouring genetic mutations, this process requires dysferlin and caveolin-3 respectively. Conversely, extensive physical and functional overload of the muscle after tenotomy [81], removal of synergic muscles or other experimentally induced/pathological conditions [82] can cause traumatic lesions inducing muscle regeneration. The entire process can be summarized in three phases: 1) necrosis of the existing myofibers and inflammatory responses; 2) activation of interstitial cells with proliferation and differentiation of muscle precursors; 3) new myofiber formation that completes the regeneration process. Necrosis of the existing damaged fibers induces the leakage of the sarcoplasm out of the fiber, resulting in an abnormal increase in plasma levels of creatine kinase [83] and miR-133a [84]. The increased permeability of the membrane is usually used as an indication of damage, such as after extensive exercise or in case of degenerative diseases. Experimentally, it is easily detectable since the membrane becomes permeable to specific dyes (Evans blue and procion orange) [85, 86]. Among, all the molecules that are lost in the blood stream, desmin could play a role as a chemoattractant following injury [87]. Moreover, the induced Ca<sup>2+</sup> imbalance activates proteolysis by calpains able to rapidly degrade many proteins including big substrates (e.g. titin and nebulin of the Z disk) [88]. Myofiber necrosis simultaneously induces activation of the complement cascade and leucocyte inflammatory response from 30 minutes to 2 days after bupivacaine (local anaesthetic markedly cardiotoxic) injection. This process is followed by the activation of macrophages [89]. In particular, neutrophils are initially recruited, and followed by two different macrophage populations that are responsible of the switch between pro-inflammatory and

anti-inflammatory environments. Indeed, pro-inflammatory monocytes sustain myoblast proliferation, while later, upon phagocytosis of debris, they switch toward an anti-inflammatory profile, promoting myoblast differentiation into myofibers [6]. Early CD68<sup>+</sup>/CD163<sup>-</sup> macrophages, that usually reach their peak in abundance around one day post injury before subsequently decreasing, are able to clear cell debris and to produce pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1. Then CD68<sup>+</sup>/CD163<sup>+</sup> macrophages become predominant up to 4 days post injury, thereby lasting till the end of the repair process. This group of cells is responsible of secreting anti-inflammatory cytokines, including IL-10. Moreover, they seem to induce SC differentiation [90]. A further step consists in the repair of the extracellular matrix (ECM) around the newly forming fibers, and to give extra-support to the muscle during contraction [91]. In normal conditions, ECM accumulation and basal lamina repair are usually under the control of extracellular proteases and their inhibitors [92]. An excessive and persistent ECM deposition (fibrosis) leads to a defect in the regenerative process. Fibroblasts are the main cell type responsible for matrix deposition: indeed similarly to SCs they can proliferate and migrate to the injury site immediately after damage [93]. Chronic inflammation plays a crucial role in severe myopathies such as DMD where endomysial fibrosis is a myopathological feature responsible of poor motility and later on fatty replacement [94]. Depending on the entity of the damage and on the conditions of the tissue, the appearance of small centro-nucleated myofibers, often positive for embryonal Myosin Heavy Chain and not always completely fused, is a common hallmark [95]. Formation of branched myofibers is classic feature of neuromuscular diseases accompanied by the presence of scar tissue [96]. Finally, when muscle regeneration is almost complete, new myofibers can undergo hypertrophy, due to contractile protein synthesis, and the single myonuclei migrate to the fiber periphery, generating functionally mature muscle fibers.

The activation of SCs is characterized by the exit from a quiescent state to a proliferative one. CD34 is highly expressed in quiescent SCs but strongly down-regulated during SC activation. This anti-adhesive molecule seems to facilitate cell migration and promotes proliferation during the early phases of SC activation [97]. Analogously, when SCs are activated, they can be directed to the site of injury due to a dynamic expression of Ephrin receptors and ligands [98]. Intracellularly, SCs shift from the expression of Pax7 to a gradual expression of the Muscle Regulatory Factors (MRFs, master regulators), namely Myf5, MyoD, Mrf4 and Myogenin, which subsequently drive the skeletal myogenesis [35]. Myf5 locus is active in almost 90% of quiescent SCs, suggesting that almost the total number of these cells is committed to the myogenic lineage [33]. Interestingly, the ratio among the levels of expression of these transcription factors is a determinant of SC fate. Indeed, Pax7 decreases MyoD transcription activity and stability, while Pax7 transcription is thereby repressed by Myogenin, once the cells are completely differentiated [99]. This is mediated by for example High Mobility Group Box-1 - Receptor for Advanced Glycation End-products (HMGB1-RAGE) axis [100]. Whether this ratio is maintained or not, impairment in regenerative capacity is observed, such as during cancer-



induced muscle atrophy [101]. One day after activation, SCs express either MyoD or Myf5 while both are coexpressed at day 2 [102]. Interestingly, *in vitro* or *in vivo* ablation of Myf5, MyoD or Mrf4 results in only mild myogenic abnormalities, while genetic ablation of all three transcription factors abolishes myogenesis [103]. MyoD-null mice display a reduced muscle mass and an impairment of muscle regeneration [104]. Analogously, when MyoD is missing, low levels of Myogenin are expressed, stressing the hierarchical importance of these transcription factors in achieving specification [105]. Conversely, the ectopic expression of MyoD in fibroblasts is sufficient to induce differentiation to myotubes underlying the crucial importance of this DNA-binding protein for skeletal muscle fate [106]. The transcriptional activity of MyoD and of the downstream Myogenin and Mef2s activates muscle specific genes for structure and functionality, including actin, myosin and troponin genes [107].

### DIRECT CONTRIBUTION OF ADULT STEM CELLS TO SKELETAL MUSCLE REGENERATION

The pivotal and non-redundant role of SCs delineates a large consensus in the scientific community of myologists regarding the fact that skeletal muscle regeneration does not occur without SCs. However, the emergence of interstitial stem cells are questioning of the precise role of SCs since also interstitial cells direct contribute to adult myogenesis and they are crucial in promoting differentiation and resolving inflammation. Physiological and pathological conditions can induce muscle differentiation that normally is addressed by SCs during extensive exercise or muscle injury [85, 86]. Indeed, the SC pool can increase during four days after a single session of exercise and can drastically reduce when exercise ceases [87]. Besides, exposure to  $\gamma$ - or X-ray radiation can block overload- or exercise- induced SC activation [88]. Interestingly, following exercise, a quantitative increase in the number of Myf5-expressing cells of approximately six fold was observed up to 96 hours after the training session, confirming that muscle SCs are the primary responders to exercise-induced stress and consequent muscle repair/adaptation [89]. From the second day after injury, the generation of Sphingosine-1-Phosphate (S1P) seems to be required, since in its absence, SCs do not proliferate and support regeneration [90]. As a bioactive lipid, S1P is able to modulate many signaling pathways, and it is involved in cell- to -cell interaction, cell survival and proliferation [91]. Recently, the sphingosine kinase/S1P axis has been recognized as critical in the migratory mechanism of SCs [92]. Moreover, S1P promotion of SCs entry into the cell cycle has been demonstrated as dependent upon STAT3 activation. In addition, the inhibition of the catabolic enzyme sphingosine phosphate lyase could suggest a therapeutic strategy for muscular dystrophies [93]. Nitric Oxide (NO) is important for SCs since it activates matrix metalloproteases able to induce the release of Hepatocyte Growth Factor (HGF). Following HGF activation of the receptor c-Met, SCs start to proliferate [94]. SC proliferation seems to be regulated by Notch and Wnt signaling, within a fine chronological pattern [95]. In addition, SCs of adult mice with abolished Notch signaling undergo spontaneous activation from the quiescent state to differentiation, resulting in depletion of the SC pool and failure of muscle regeneration [95]. Moreover, the ab-

sence of Hesr1 and Hesr3, Notch signaling downstream target genes, usually expressed in quiescent SCs, induces reduction of SC self-renewal and depletion of the SCs pool, leading to the consequent impairment of muscle regeneration [96]. Wnt signaling pathway is necessary for muscle differentiation and for myogenic commitment of stem cells, and it is also involved in fibrosis during aging [77]. Wnt induces the differentiation of proliferating myoblasts if activated at a correct time, since premature Wnt activation induces an anticipated but not sufficient differentiation stimulus [97]. Thus, *in vivo* Wnt7a injection into injured muscles contributes to the myogenic program as reported by an increase in muscle mass and cross sectional area [98]. It is possible that in regenerating muscles, Wnt/ $\beta$ -catenin pathway increases Wnt7a production and activation of SCs, with replenishment of SC pool [21]. This could be the reason why recently, *ex vivo* stimulation of freshly isolated SCs with Wnt7a for a few hours resulted in amelioration of the migration ability of these cells when injected into new mice. Indeed, the transplantation resulted in a massive increase in engrafted cells and a hypertrophic effect upon the newly formed myofibers, with higher muscle force [99]. Furthermore, Wnt/ $\beta$ -catenin downregulation, modulated by Wnt10b, a protein of the Wnt-family, induces an increase in adipogenic differentiation in the elderly [100]. According to this observation, Wnt10b-null mice show excessive lipid accumulation during muscle regeneration [101]. Further studies are needed in order to shed a light on the complexity of Wnt signaling and its ability to induce adipogenic and myogenic differentiation.

Myofibers secrete SDF-1, a migration factor that binds CXCR4 receptor on SCs, influencing their movement [28]. Moreover, after injury, the Notch ligand Delta has been found upregulated on myofibers, thus able to activate proliferation of quiescent SCs. Interestingly, in the same study, the reduction in Notch signaling could compromise SC activation and muscle regeneration [102]. The entire regenerative process is also promoted by a series of growth factors, present in the interstitium or in the serum, that can extend or make effective the induction of SCs towards differentiation. The overexpression of pro-migratory stimuli like MM9 or VEGF in skeletal muscle promotes angiogenesis and improves the engraftment of transplanted myoblasts [103]. HGF mediates different processes, including cell proliferation, migration and motility. In skeletal muscle, HGF function is mainly characteristic of the early phases of regeneration, since muscle injury would increase NO levels and activate the MMP responsible for its proteolytic cleavage [104]. HGF acts through its receptor c-Met, usually expressed by both quiescent and proliferative SCs, stimulating the entrance in cell cycle [27] and inhibiting cell differentiation through a sustained MAPK/ERK signaling [105]. Mice expressing an engineered protein (Met-Activating Genetically Improved Chimeric Factor-1 or Magic-F1) with two repeated Met-binding domains display muscle hypertrophy and SCs are protected from apoptotic events [106]. FGFs have been reported to affect skeletal muscle regeneration. FGF6<sup>-/-</sup> mice show impaired regeneration, with reduction of MyoD and Myogenin expression cells, and accumulate scar tissue and collagen deposition in skeletal muscle tissues [107]. *In vitro*, FGF-1 inhibition induces myogenic differentiation, while exogenous FGFs

stimulate proliferation of rat primary myoblasts [108]. Interestingly, it has been shown that *alpha-Sarcoglycan* (*Sgca*) deficiency could cause reduction in FGF-R1 expression and consequently impairment of SC proliferation in response to basic Fibroblast Growth Factor (bFGF) [109]. SC activity is also regulated by Insulin like Growth Factor (IGF-1) and IGF-2 [110] that induce activation of MRFs [111]. Two main signaling pathways are activated downstream of IGFs. The phosphatidylinositol 3-kinase (PI3K) pathway, that elicits an antiapoptotic effect mainly mediated through AKT/ mTOR [112] and the AKT/ p38-MAPK pathway that is involved in myoblasts differentiation [113, 114]. The Ras/ Raf/ extracellular response kinases (ERK) signaling cascade is also crucial for SC proliferation [115]. Notably, enhanced myoblast proliferation is obtained after stimulation with mechano-growth factor (MGF), which is derived by alternative splicing of the IGF-1 gene [116]. Interestingly, co-injection of myoblasts and IGF-1 or bFGF was able to double the migration distance of the cells, although without increasing SC engraftment [117]. Indeed, the poor transendothelial migration abilities of SCs still remain one of the major limiting factors that make SCs inadequate for systemic delivery. Moreover, SCs easily undergo senescence after isolation, expansion and manipulation and result in a reduced engraftment due to immune rejection and poor survival [118]. For all of these reasons, novel candidate progenitors with myogenic proliferative and migratory capabilities are increasingly necessary.

### Pericytes and Skeletal Muscle Regeneration

To date, several studies have demonstrated the ability of pericytes to differentiate to skeletal muscle giving rise to functional muscle fibers and smooth muscle layer of blood vessels [119-121]. The contribution of pericytes to skeletal muscle varies among different muscles and is enhanced after acute injury or in the presence of chronic disease. However, in chronic conditions pericytes seem to mainly contribute to fibrosis as observed in type I diabetic and nephropathy, which implies conversion of pericytes into myofibroblasts [122]. Interestingly, AP<sup>+</sup> cell increment in biopsies coming from different dystrophic patients could be interpreted as an attempt to regenerate muscle tissue [123]. Lately, two different pericyte populations (type-1 and -2) have been distinguished and their commitment and complete differentiation to skeletal muscle have been correlated to the expression of Nestin [121]. Indeed, type 1 pericytes, negative for the Nestin marker, seem to contribute to the deposition of collagen in old mice [124]. Nevertheless, further studies are needed to determine if the absence of the specific type-2 subpopulation can really determine impairment in skeletal muscle regeneration. Also, to identify whether other markers are selectively expressed on the two different populations that can be employed to selectively isolate type-2 pericytes and further investigate a possible applicability of these cells.

### MABs and Skeletal Muscle Regeneration

Intrinsic properties of migration and survival while in the circulation and in their high efficiency of transplantation inducing high regeneration abilities have been observed in

MABs. Indeed, injection of *Sgca*-null MABs, after correction for the missing protein into *Sgca*-null mice (mouse model used for LGMD) gave rise to *Sgca*<sup>+</sup> myofibers [53]. Moreover, Human MABs injected in the femoral-artery of mice with combined immune deficient-X- linked muscular dystrophy (*scid-mdx*) gave rise to dystrophin<sup>+</sup> myofibers [50]. In addition, several factors can ameliorate the migration from blood vessels towards the injured tissue and the engraftment of MABs, like HMGB1, SDF-1, CXCR4 and  $\alpha$ 4-integrin [125]. Similarly nitric oxide pretreatment of MABs can improve their therapeutic potential [126]. Recently, technological improvements for gene correction as Piggy-Bac-mediated approaches make MABs more suitable for further studies in muscle regeneration [127]. Moreover, gain and loss of function experiments have revealed that Delta-like ligand 1 (Dll1)-activated Notch1 and Mef2C supports MAB commitment *in vitro* and induces a strong amelioration of their engraftment *in vivo* [128]. Finally, preliminary data from Phase I/II clinical trials using HLA-identical allogeneic MABs (EudraCT number 2011-000176-33) indicate safety and presence of donor derived DNA and dystrophin in treated patients (Table 2). Further studies still need to be done, in order to understand if other pathways, such as BMP- and Wnt signaling can be involved in myogenic potential and cell engraftment, in order to corroborate MAB regenerative potential.

### PIC Cells

PIC myogenic potential has been proved *in vitro* [63]. Moreover, when these cells are injected in injured muscles, surface marker analysis has revealed that these cells are able to repopulate not only the interstitial fraction of PIC cells, but as well, the fraction of Pax7<sup>+</sup> cells, letting space for speculation about the myogenic potential of these cells in the adult [63, 80]. Some other issues on the possible origin of PICs have been proposed and are of significant interest, since some of the markers of PICs and FAPs are in common (CD34<sup>+</sup>/ Scd1<sup>+</sup>/ CD45<sup>-</sup>) and these two cell types share the same localization (Fig. 1). Quite recently, PICs were demonstrated to differentiate to adipose cells [129], opening the discussion on the possible overlap between interstitial populations such as FAPs and PICs. How the Pw1 transcription factor is silenced in the adipocyte cell conversion is still under investigation.

### MDSCs and Skeletal Muscle Regeneration

As the MABs, intra-arterial transplantation of MDSCs contributes to regenerate myofibers [130] and differently from MABs they give rise to endothelial and neural cells [65]. Moreover, MDSCs have also the capability to repopulate the hematopoietic pool in lethally irradiated mice, inducing muscle repair [131]. The relationships between MDSCs, MABs and other myogenic cells are still unknown and further translational investigation is necessary to better understand the therapeutic potential of MDSC.

### SPs and Skeletal Muscle Regeneration

SPs have been induced to undergo myogenic commitment by co-culturing them together with myoblasts or forcing Pax7 or MyoD expression [132]. A subpopulation of SPs



Table 2. A partial list of interventional trials for muscular dystrophies based on adult stem cell treatments.

Study (Code)	Purpose	Phase [Patient Number]	Intervention [Dose]	Ages Genders	Status	Locations
<b>Safety and efficacy of bone marrow derived autologous cells (NCT01834066)</b>	Safety and efficacy of bone marrow derived autologous cells	Phase1/2 (25)	Intravenous delivery (100 MIO)	6 -25 y male/female	Recruiting	India
<b>Efficacy of umbilical cord MSCs (NCT02285673)</b>	Efficacy of umbilical cord MSCs	Phase1/2 (10)	NA	7-20 y male	Recruiting	Turkey
<b>Allogeneic human umbilical cord MSCs (NCT02235844)</b>	Muscle regeneration and anti-inflammatory properties of umbilical cord MSCs	Phase1 (1)	NA	28-31 y male	Enrolling by invitation	USA
<b>Stem Cell Therapy in DMD (NCT02241434)</b>	Autologous bone marrow mononuclear cell therapy	Phase1 (500)	NA	3-25 y male/female	Recruiting	India
<b>Safety and efficacy of BMMNC for DMD patients (NCT01834040)</b>	Single arm, single centre trial to check the safety and efficacy of BMMNC transplantation	Phase1/2 (30)	Intravenous delivery (100 MIO)	4-20 y male/female	Recruiting	India
<b>Intra-arterial delivery of HLA-identical allogeneic MABs (EudraCT 2011-000176-33)</b>	Safety and improvement of muscle strength of local and systemic adverse events	Phase1 (6)	Intra-arterial delivery (escalating dose)	< 18 y male	Active, not recruiting	Italy
<b>Cell therapy in limb girdle muscular dystrophy (LGMD) (NCT02245711)</b>	Effects of stem cell therapy on LGMD patients	Phase1 (200)	Intrathecal delivery (NA)	15-60 y male/female	Recruiting	India
<b>Intramuscular transplantation of MSCs in patients with facioscapulohumeral dystrophy (FSHD) (NCT02208713)</b>	Evaluation of safety and feasibility of muscle and adipose derived MSCs in FSHD	Phase1 (15)	Intramuscular delivery (NA)	18-50 y, male/female, no volunteers	Recruiting	Iran

characterized by CD45<sup>-</sup>/ Sca-1<sup>+</sup>/ ABCG2<sup>+</sup>/ Pax7<sup>+</sup>/ Syndecan-4<sup>+</sup> marker profile has been found to differentiate into myofibers and undergo self- renewal in the quiescent compartment after *in vivo* injections [45]. Importantly, these cells can engraft and restore the levels of dystrophin when intravenously injected into mdx-mice [45, 133].

### SK-34 and Skeletal Muscle Regeneration

Sk-34 cells have also been successfully transplanted *in vivo* and have demonstrated great differentiation potential not only towards the skeletal muscle tissue, but as well towards the endothelial and the Schwann cells [134]. Apparently these cells are even able to undergo differentiation towards cardiomyocytes and, when injected into the infarcted heart, improve left ventricle functionality [135]. The great utility of these cells indeed is their ability to give rise to different lineages. Many questions remain to be solved in order to understand the best way to apply them in preclinical and clinical trials however.

### Circulating Bone Marrow Cells and Skeletal Muscle Regeneration

Intramuscular and intravenous injections of bone marrow derived cells into injured muscles have demonstrated the

possibility for these cells to regenerate skeletal muscle [44], even if the ability to reconstitute the SC niche was considered low. Nevertheless, systemic injection into irradiated mice could reconstitute the Myf5<sup>+</sup>, c-Met<sup>+</sup> and  $\alpha$ 7integrin<sup>+</sup> SC pool [136]. Adult bone marrow-residing hematopoietic stem cells, isolated for the positivity for c-Kit/ Sca-1/ CD45 in different association, are all able to fuse into newly formed myofibers [137]. Interestingly, muscle stress and injury could be two major stimuli to improve engraftment of bone marrow cells into regenerating muscles [138], providing further proof that the microenvironment can guide cell specification. Indeed, bone marrow cells have been demonstrated to depend on SDF-1/ CXCR4 signaling, chemokine receptor 2 (CCR2), and on the Hepatocyte Growth Factor (HGF)/c-Met axis [28].

Mesenchymal Stem Cells (MSCs) have been characterized as CD29<sup>+</sup>/ CD44<sup>+</sup>/ CD71<sup>+</sup>/ CD73<sup>+</sup>/ CD90<sup>+</sup>/ CD105<sup>+</sup>/ CD106<sup>+</sup>/ CD166<sup>+</sup>, CD34<sup>-</sup>/ CD45<sup>-</sup> and have been cultivated *in vitro* as adherent growing cells able to differentiate under specific conditions to various lineages, including osteogenic, chondrogenic, adipogenic [139]. Further progress in developing strategies to enhance MSC commitment toward skeletal muscle will no doubt arise through research into new and important signaling pathways. Indeed, co-culture of MSC

and C2C12 myoblasts have reported to induce Notch signaling pathway and VEGFR phosphorylation and expression [140]. Moreover, the same research group has recently demonstrated that S1P, a natural bioactive lipid that binds to a family of five G protein-coupled receptors, is secreted by MSCs and is one of the stimulatory effectors of these cells on C2C12 myoblast and SC proliferation [141]. Nevertheless, despite the exciting results in preliminary pre-clinical studies with BM-MSCs, there are still several unanswered questions and we are currently in the dark about their clinical relevance.

### HSCs and Skeletal Muscle Regeneration

From human adult peripheral blood, a fraction of mononucleated cells (CD133<sup>+</sup>) shows myogenic potential when co-cultured together with myoblasts or in presence of Wnt7a secreting fibroblasts [142]. Indeed these cells, which are able to repopulate the bone marrow niche and to differentiate into endothelial cells, have been injected intramuscularly in scid/mdx mice, giving rise to dystrophin positive myofibers [142]. Surprisingly, human M-cadherin and Myf5 genes were detected in the undifferentiated compartment of these muscles, testifying the ability for these cells not only to engraft but as well to replenish the SC niche [142]. After these experiments, CD133<sup>+</sup>/CD34<sup>+</sup> cells have been identified in skeletal muscle biopsies of different musculopathies, such as dermatomyositis, polymyositis and inclusion body myositis [143]. Human muscle-derived stem cells expressing hematopoietic markers like CD133 have been injected intramuscularly in cryo-injured Rag2<sup>-/-</sup> gamma<sup>-/-</sup> C5<sup>-/-</sup> mice (an experimental model of muscle degeneration), increasing regeneration [144]. These cells have also been employed to treat scid/mdx mice [145], and in an autologous setting in a phase I clinical trial (Table 2) resulting in an increased number of capillaries associated with muscle fibers and a slow to fast fiber switch [146]. Recently, antisense oligonucleotide-based experiments have demonstrated a partial rescue of dysferlin levels in CD133<sup>+</sup> stem cells isolated from patients with Miyoshi myopathy and subjected to myogenic induction. In addition, dysferlin-transduced CD133<sup>+</sup> stem cells were able to affect positively the dystrophic phenotype of transplanted scid/blAJ dysferlin-null mice [147].

## INDIRECT CONTRIBUTION TO SKELETAL MUSCLE REGENERATION

### FAPs and Skeletal Muscle Regeneration

As already reported, FAPs are not able to give rise to skeletal muscle cells. However, they are crucial for the paracrine modification of the skeletal muscle niche to modulate adult myogenesis. The activation of IL-4/IL-13 signaling induces FAP proliferation and supports myogenesis, inhibiting the adipogenic potential of these cells [148] [149]. Recently, it has been demonstrated that glucocorticoids can further worsen muscle degeneration, since dexamethasone administration blocks IL-4 secretion and stimulates adipogenic commitment of FAPs [150]. Moreover, it has been hypothesized that Nitric-Oxide (NO) not only improves regeneration but reduce the number of PDGFRα<sup>+</sup> cells, the synthesis of connective tissue and deposition of fat into the skeletal muscle tissue in dystrophic muscle. Consistently NO

inhibits FAP adipogenic differentiation through increased expression of miR-27b [151]. Finally, epigenetic-modifying drugs have been revealed to be interesting tools to influence FAP differentiation fate decisions. Indeed, the BAF60c-myomiR axis has been identified as a key mediator of HDAC inhibitor-induced changes in FAP plasticity [152]. The mechanisms that induce the changes in chromatin structure and that permit the activation of the myomiR/BAF60c network still need to be clarified.

### TCs and Skeletal Muscle Regeneration

Another supporting role in muscle regeneration is apparently played by the interstitial telocytes, expressing PDGFRα and c-kit markers. They release VEGF and are able to form networks. Moreover, all of these characteristics have been confirmed in human TCs, suggesting their clinically relevant role in skeletal muscle regeneration and repair, at least after trauma [81]. Further studies will be needed in order to clarify the function of these cells and their interaction with the local milieu and with other cells.

### Tcf4 Positive Fibroblasts and Skeletal Muscle Regeneration

Tcf4 positive fibroblasts have an important paracrine effect during adult myogenesis, since ablation of SCs not only impairs muscle regeneration but also interferes with Tcf4 fibroblast function and leads to an increase in connective tissue. This is a mutual phenomenon since the lack of Tcf4<sup>+</sup> fibroblasts results in a premature SC differentiation leading to abnormally small regenerated myofibers [85]. Further studies need to be conducted in order to identify the signaling pathways that can orchestrate such a strict communication between Tcf4 positive fibroblasts and SCs.

## GENE EXPRESSION MODIFICATION FOR CELL-BASED THERAPIES

MPCs from healthy donors have been extensively proposed as the main sources for cell-based therapies in MDs. Unfortunately, poor survival and migration of MPCs and also possibly an immune response to donor represents significant barriers to therapeutic development [153]. *Ex vivo* gene therapy allowed delivery of therapeutic genes into MPCs before transplantation and has been proposed as a treatment for DMD. Since full-length dystrophin transferring showed several methodological issues because of its size (14 kb), several alternative strategies have been explored. A nonviral approach combining nucleofection and the phiC31 integrase provided a safe site-specific integration of the transgene [154]. However, adeno-associated virus (AAV) and lentiviral vectors were so far the main proposed tools for micro- or mini-dystrophin transduction in MPCs. Nevertheless, the lack of genomic integration of AAV and the scarce lentiviral transduction in myofiber cultures directly limit their therapeutic potential. In addition, although lentiviral vectors guarantee genome integration and stable expression [155], they have a maximum of 10 kb cloning capacity, precluding their use for delivering the full-length 14-kb dystrophin cDNA. Thus an exon-skipping strategy was introduced in *ex vivo* gene therapy as potential treatment of DMD. Exon-skipping acts by RNAs (*i.e.* U7) or DNAs able to inter-

ferre with the normal splicing and restore the open-reading frame of mutated dystrophin. In this way exon-skipping technology induces the splicing machinery to skip the mutant exons in missense, nonsense, and eventually translationally silent mutations. In 2007 Quenneville and colleagues demonstrated the applicability of lentiviral-based *ex vivo* gene therapy using modified DMD-derived MPCs with microdystrophin expression or U7 based exon-skipping strategies in both the mdx mouse and in non-human primates [156]. The AAV-mediated exon-skipping strategy has also been adopted in genetic correction of CD133<sup>+</sup> myogenic progenitors isolated from both skeletal muscle tissue and blood of DMD patients. Intra-arterial transplantation of these cells in scid/mdx mice showed an appreciable rescue in dystrophin expression, consequently restoring muscle structure and functionality [145]. Recently it has been shown that culture of human muscle fiber fragments can be used as a source of SCs which are genetically manipulable for the expression of the myogenesis regulator Pax7 through Sleeping Beauty transposon-mediated nonviral gene transfer [157]. Since entire fragments can be implanted *in vivo*, this method highlights the possibility of combining gene expression modification with a high efficiency of human satellite cell transplantation in an animal model for muscular dystrophies [157].

TALEN (transcription activator-like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeats) genome-editing approaches provide an efficient strategy for correcting point mutations. TALENs are endonucleases with a TAL effector DNA binding domain and a DNA cleavage domain able to induce a double strand break (DSB) in the DNA followed by homologous recombination. CRISPR is an RNA-guided gene-editing system able to introduce a double strand break by delivering the Cas9 protein and appropriate gene specific guide RNAs. CRISPR/Cas9-mediated genome editing was recently used to correct the mutation in dystrophin exon 23 in the germ line of mdx mice [158]. Using three correction methods (exon skipping, frameshifting, and exon knockin) in DMD-patient-derived iPSCs TALEN and CRISPR-Cas9 technologies were employed to restore the expression of dystrophin [159]. In this study, Li *et al* showed that exon knock-in was the most effective approach and TALEN and CRISPR-Cas9 corrected iPSCs were able to differentiate into skeletal muscle cells and express the full-length dystrophin protein. These are very promising gene editing technologies that will be further developed and so far no clinical trials are yet planned.

#### CELL-BASED THERAPIES IN MUSCLE REGENERATION: ADVANCES AND LIMITATIONS

According to the type and the stage of the muscular disorder, the failure in muscular regenerative capability is an important determinant of muscle wasting [160]. Inflammatory cells, from different origins, can strongly participate in the events underlying muscular disruption occurring in chronic degenerative diseases, such as in dystrophinopathies, or in a mild way as observed in cancer-related muscle wasting. These cells, together with the prominent effects mediated by both local and circulating pro-inflammatory mediators, can contribute to the altered muscle regeneration capability observed in the majority of muscular diseases. Overall,

chronic local inflammation and hampered regeneration potential lead to the increased susceptibility to damage in muscle fibers that become atrophic and replenished by fibrotic tissue. Many efforts have been made aiming to treat severe muscular illness using a cell-based therapeutic approach, especially in MDs [160]. Skeletal muscle-resident progenitors cells are the most adopted sources experimented so far including SCs, pericytes, FAPs, PICs, MDSCs and SP, while among interstitial stem cells, MABs have been investigated as potential candidates for therapeutic approaches, in the treatment of sarcoglycanopathies and dystrophinopathies. Among the most common muscular degenerative diseases, MDs are indeed characterized by both impaired myogenic stem cell function and extreme deposition of extracellular matrix [161]. The early-aged onset, the premature death, the decreased quality of life of the patient and the high socioeconomic burden [162] together with limitations related to human myoblast transfer therapy, have encouraged the investigation of stem cell sources other than SCs. Thus, the research in cell-based therapies for treatment of muscle degeneration has been oriented toward MABs and pericytes. These alternative sources of cells are more abundant, more accessible and show a better motility after transplantation compared to SCs [157]. MABs show a strong ability to cross the vessel wall and intra-arterial delivery of mice lacking  $\alpha$ -SG, a limb-girdle model of muscular dystrophy, demonstrated promising results in amelioration of the dystrophic phenotype [53]. Furthermore, recent advances in skeletal muscle tissue engineering showed that the use of specific scaffold tissues supporting progenitor/stem, such as polyethylene glycol (PEG)-fibrinogen [163], ameliorates both survival and engraftment of transplanted MPCs [164]. Cell-based therapies for muscle disorders still represent a road under construction and likely all of these emerging technologies will be combined together to test novel therapeutic strategies [160].

#### MIRNAS AND EXOSOME

In the last decade, many high level publications showed the critical roles of micro-RNAs (miRNAs) in regulating biological phenomena, including stem cell fate and skeletal muscle regeneration. MiRNAs are non-coding RNAs able to modify gene expression through altering mRNA stability or inhibition of translation. miRNAs sit in the genome either as individual transcriptional units or are co-transcribed as intronic sequences of other genes [165, 166]. The biogenesis of miRNAs consists in the generation of pri-miRs by RNA polymerase II that are processed by the microprocessor complex into pre-miRs [167]. Exportin is responsible for pre-miRs transportation to the cytosol and Dicer cleaves them into ~22nt-long double-stranded molecules. The guide strand is on the RNA-induced silencing complex (RISC) that is able to carry out gene silencing by recognizing 3' untranslated region (3'-UTR) of target mRNAs [168]. Several miRNAs have been shown to be involved in skeletal muscle homeostasis controlling embryonic and adult myogenesis. Recently, they have also been proposed as diagnostic markers in DMD serum derived patients (NCT02109692; Table 2). Mir-206, MiR-1 and miR-133 represent the myomiRNA family, since they are highly expressed in skeletal muscle tissue and regulate its regeneration [169]. Evidence

is also accumulating for broadly expressed miRNAs that display a critical effect on muscle remodeling. Mir-24 [170] and mir-29 [171, 172], for instance, are ubiquitously expressed but they have an impact on myogenesis by repressing TGF $\beta$  signaling and the Polycomb silencing complex respectively. In addition, mir-29 can target the tyrosine kinase Akt3, associating it with growth factor signaling [173]. A remarkable example of miRNA-mediated cell fate shift consists of miR-669a/q, able to control MyoD expression in murine cardiac progenitor cells. *Scgb*-null mice show impairment in miR-669a/q expression resulting in aberrant differentiation of cardiac MABs that can be rescued by AAV miR-669a-based therapy [174, 175]. Although further studies are necessary to better interpret the role of miRNAs in adult stem cell biology and skeletal muscle regeneration, miRNAs are definitely key players in muscle regenerative medicine. Another open question is regarding how miRNAs can mediate cell-cell interactions. Emerging literature is focusing on the role of exosomes, extracellular vesicles containing proteins and RNAs including miRNAs [176]. Exosomes released during muscle cell differentiation contain myomiRNAs and paracrine action of exosomes secreted by stem cells has been also reported, suggesting a key role for exosomal miRNAs in intracellular communication. Mesenchymal stem cells release exosomes that activate angiogenesis and myogenesis pathways facilitating skeletal muscle repair. MyomiRNAs likely carried by exosomes are detected in body fluids of animal models of muscle degeneration and their presence was further confirmed in patients affected by muscular dystrophies [177]. Exosome-miRNAs are now considered putative biomarkers in muscle degeneration/regeneration and are providing a new impetus for the exploration of novel therapeutic approaches for muscular degeneration [177].

## CHALLENGES AND FUTURE PERSPECTIVES

Promising results have been obtained from the transplantation of engineered mouse embryonic stem cells (ES) conditional expression of Pax3 and Pax7. ES Pax3/Pax7 showed high ability to engraft in the satellite cell niche through systemic injections, prolonged self-renewal predisposition and an excellent response to injury. Although this approach still did not reach the clinic, these findings support the therapeutic relevance of ES-derived myogenic progenitors for skeletal muscle degenerative conditions [178]. Moreover, several studies showed that myogenic progenitors generated from induced pluripotent stem (iPS) cells are also able to participate in muscle regeneration and correct the dystrophic phenotype [179-182]. Further studies on the causal relationship of exosome-mediated processes are crucial in order to better delineate their involvement in striated muscle degeneration/regeneration. Safety studies on the use of pluripotent stem cell derivatives are also desirable. It is reasonable to predict that more studies are expected on myogenic precursors generated from pluripotent stem cells in combination with miRNA and novel gene editing technologies to treat muscle degeneration. Nevertheless, the results of those studies will require a deep comparison with those performed using adult stem cells, the 'gold standard' for stem cell-based protocols in the last decades.

## ABBREVIATIONS USED

CXCL	= Chemokine Ligand
DMD	= Duchenne Muscular Dystrophy
ECM	= Extra Cellular Matrix
FAPs	= Fibro/Adipocyte Progenitors
HGF	= Hepatocyte Growth Factor
HSC	= Hematopoietic Stem Cells
IFN- $\gamma$	= Interferon- $\gamma$
IGF	= Insulin like Growth Factor
IL	= Interleukin
LGMD2A	= Limb Girdle Muscle Dystrophy-type 2 A
MABs	= mesoangioblasts
MAPKs	= Mitogen Activated Protein Kinases
MDSC	= Muscle Derived Stem Cell
MGF	= Mechano Growth Factor
miRNA	= micro RNA
MMP	= Matrix Metalloproteinase
MRFs	= Myogenic Regulatory Factors
MSC	= mesenchymal stem cells
MyHC	= Myosin Heavy Chain
NF-kB	= Nuclear Factor kappa b
NG2	= Neural-Glial-2 chondroitin sulphate proteoglycan
NO	= Nitric Oxide
NOS	= Nitric Oxide Synthase
PICs	= Pw1 expressing interstitial cells
PI3K	= Phosphatidylinositol 3 kinase
SC	= satellite cells
Sk-34	= Skeletal muscle-derived CD34+/45- cells
SP	= Side Population
S1P	= Sphingosine-1-phosphate
TCs	= Telocytes
TGF $\beta$	= Tumor Growth Factor beta
TNF $\alpha$	= Tumor Necrosis Factor alpha

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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